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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 :
A61K 38/17, 39/395, A61P 37/06, 27/02, 29/00

A2

(11) International Publication Number: **WO 00/27421**

(43) International Publication Date: 18 May 2000 (18.05.00)

(21) International Application Number: PCT/US99/26262

(22) International Filing Date: 5 November 1999 (05.11.99)

(30) Priority Data:
60/107,351 6 November 1998 (06.11.98) US

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(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: LOCAL USE OF SOLUBLE TUMOR NECROSIS RECEPTOR I (sTNFRI) FOR PROPHYLAXIS AND TREATMENT OF CORNEAL TRANSPLANT REJECTION AND OTHER DISORDERS OF THE EYE

(57) Abstract

Topical application of soluble tumor necrosis factor receptor antagonist (sTNFRI) is shown to promote corneal transplant survival in a murine model of orthotopic allotransplantation, having a significant effect in prolonging graft survival. Furthermore, the promotion of graft survival is associated with a significant decrease in corneal inflammation. Therefore, sTNFRI and related antagonists to tumor necrosis factor–α activity can be used in a therapeutic composition for local prophylaxis and treatment of allograft rejection and a wide array of immunogenic inflammatory diseases of the eye. The composition comprises a therapeutically effective amount of a tumor necrosis factor–α antagonist in association with a pharmaceutically acceptable carrier vehicle for local application.

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LOCAL USE OF SOLUBLE TUMOR NECROSIS RECEPTOR I (sTNFRI) FOR PROPHYLAXIS AND TREATMENT OF CORNEAL TRANSPLANT REJECTION AND OTHER DISORDERS OF THE EYE

FIELD OF THE INVENTION

This invention relates to the prophylaxis and treatment of corneal transplant rejection and other immune and inflammatory disorders of the eye and more particularly to a topical treatment therefor.

GOVERNMENT RIGHTS

Part of the work leading to this invention was carried out with United States Government support provided under Grant No. EY00363 from the National Eye Institute. Therefore, the U.S. Government has certain rights in this invention.

BACKGROUND OF THE INVENTION

Corneal transplantation has emerged as the most common and successful form of solid tissue transplantation with over 40,000 cases performed in the United States alone (Collaborative Corneal Transplantation Studies Research Group). In uncomplicated first allografts performed in avascular beds, the 2-year survival rate is over 90% (Niederkorn, 1990). The extraordinary success of penetrating keratoplasty can be attributed to various features of the normal cornea and anterior segment that in the aggregate account for their "immune-privileged" state (Streilein, 1995) including: (a) the avascularity of the stroma, (b) the absence of corneal lymphatics, (c) the rarity of indigenous professional antigen-presenting Langerhans cells (LC) or macrophages in the normal graft bed, (d) a unique spectrum of locally produced immunomodulatory cytokines

that suppress immunogenic inflammation and complement activation (to which the cornea itself contributes), and (e) expression of Fas ligand by these ocular tissues that can directly suppress immunogenic inflammation (Griffith et al., 1995).

In spite of the overall success with corneal transplantation, however, a substantial percentage of corneal grafts experience at least one rejection episode. This is significant since of all the technical and tissue parameters that can affect final graft outcome, immunologic rejection represents the principal threat to allograft longevity regardless of the degree of allodisparity (Mader et al.; Coster; Maguire et al.; Williams et al.; Alldredge et al.).

The advent of corticosteroids and their use in the prophylaxis and treatment of corneal transplant rejections has represented the most significant contribution to the prolongation of corneal transplant survival over the last several decades (Wilson et al.; Hill et al.). However, the local use of corticosteroids, or alternative general immunosuppressants, is associated with significant complications such as infection, cataracts, glaucoma and corneal thinning (Raizman; Hemady et al.; BarraquerFrangie et al.). General immunosuppressive therapy, when used systemically, may be associated with serious side-effects and multiorgan dysfunction (morbidity) which does at times culminate in death. It is therefore apparent that development of molecular strategies that can specifically target a critical step in the transplant rejection process is desirable and would prove to be an effective modality of circumventing the problems inherent in non-specific immune suppression.

SUMMARY OF THE INVENTION

Langerhans cells (LC) belong to the dendritic cell family and mediate antigen presentation in the cornea and ocular surface. Hence, they are capable of activating T cells and initiating ocular immune responses (Gillette et al.). Under normal physiological conditions, the central cornea is devoid of LC. However, a number of corneal stimuli (e.g., trauma, infection, cauterization) can induce centripetal migration of LC into the cornea from the limbus, the border between cornea and conjunctiva, where they may initiate antigen processing (Williamson et al.; McLeish et al.).

In the setting of corneal transplantation, the presence of LC in the donor cornea has been shown to effect host allosensitazation and graft rejection (Niederkorn, 1995). Non-corneal solid organs sensitize their hosts primarily through the "direct" pathway of sensitization where "passenger cells" from the donor emigrate to the host's lymphoid organs and activate T cells. This is known as *direct* sensitization. However, two features of the cornea, (1) its depressed expression of class II antigens, and (2) lack of "passenger cells," make this pathway less operative in the setting of corneal transplantation. Hence, sensitization of the host in corneal grafting requires the participation of host antigen-presenting cells, in a process known as *indirect* sensitization (Sano et al., 1997a).

Since in clinical corneal transplantation patients receive central corneal buttons devoid of LC, it is believed that the "indirect" pathway for corneal allograft recognition may involve activation of migration of recipient LC from the limbus to the donor corneal tissue where they can acquire foreign antigen (Sano et al., 1997b). Two lines of indirect evidence suggest that LC migration is a critical element in host

allosensitization. First, the number of infiltrating host LC in the graft bed is predictive of the swiftness with which the host acquires donor-specific delayed type hypersensitivity (Yamada et al.), and the promotion of corneal allograft survival by IL-1 receptor antagonist (IL-Ira) has been correlated with suppression of LC migratory capacity (Dana et al., 1997). Beyond these observations in experimental models of corneal transplantation, migration of limbal LC into the cornea has been associated with loss of ocular immune privilege (Dana et al., 1998) and other immunoinflammatory events in the cornea such as development of herpetic keratitis (Jager et al., 1991, 1992a, 1992b, 1995; Hendricks et al.).

The mechanisms involved in regulation of corneal LC migration are incompletely understood. Several cytokines have been implicated but only the role of IL-1 has been extensively studied (Niederkorn et al, 1989; Niederkorn, 1995; Dana et al., 1998). However, recognition of the close cross-regulation of IL-1 and TNF-α in multiple models of inflammation, and the fact that stimulation of central corneal tissue results not only in IL-1 but also in TNF-α expression from resident epithelial cells (Sekine-Okano et al.) suggested that TNF-α could be a candidate for study in regulation of corneal LC migration.

TNF-α is a pleiotropic cytokine that mediates a large number of proinflammatory functions such as up-regulation in the expression of adhesion and costimulatory molecules, neutrophil activation, induction of chemokine secretion and activation of the NF-κB signal transduction pathway (Le et al.; Eigler et al.). TNF-α activity is regulated by two distinct receptors, the type I receptor (p55) and the type II receptor (p75), which have largely homologous extracellular domains but distinct intracellular domains that can mediate discrete

cellular responses (Peschon et al.; Tartaglia et al.). It might be expected, therefore, that interfering with the binding of TNF-α to one or both of the TNF-α receptors (e.g., by using a soluble form of the receptor) would interfere with TNF-α activation of corneal LC migration. On the other hand, sTNFR has been shown to be effective primarily in immune conditions mediated by antibodies or immune complexes (antigen-antibody complexes), such as rheumatoid arthritis. It has been conclusively demonstrated that antibody responses do not play an important role in mediating the rejection of corneal transplants (Goslings et al.).

Nevertheless, it has surprisingly been found, and is reported here, that direct application of sTNFRI to corneal allografts leads to a significant prolongation of transplant survival. The results described below demonstrate that sTNFRI administration has a significant positive effect in suppressing corneal LC migration, suppressing ocular chemokine gene expression and promoting survival of allogenic corneal transplants.

Therefore, the invention is directed to a method for treating allografts and preventing allograft rejection, or for generally treating an immune or inflammatory response of the eye. In other aspects, the method also is directed specifically to suppressing corneal LC migration or to suppressing ocular chemokine gene expression. The method of the invention includes direct, local administration (e.g., by topical application or local injection or irrigation) of a therapeutic composition to an affected area of an eye of a patient. The therapeutic composition useful in the method of the invention comprises a therapeutically effective amount of a tumor necrosis factor-α (TNF-α) antagonist in association with a pharmaceutically acceptable carrier vehicle for local application. Furthermore, the therapeutic composition can be packaged as an article of

manufacture of the invention that includes a label indicating the use of the composition in the method of the invention. Preferably, the TNF-α antagonist is a tumor necrosis factor receptor and, most preferably, one of the naturally occurring (or recombinant) human isoforms, or portions thereof, soluble tumor necrosis factor receptor I (sTNFRI) or soluble tumor necrosis factor receptor II (sTNFRII). Alternatively, other TNF-α antagonists may be utilized for the same effect. These include, but are not limited to, (1) modifications of native soluble receptors that would, e.g., render these compounds more bioactive, or (2) other TNF-α antagonists that would bind and hence render inactive the TNF-α receptors (e.g., anti-TNF-α receptor antibodies) and/or (3) other soluble form(s) of the TNF-α receptors that would bind TNF-α (or its analogue lymphotoxin α) and prevent their binding to TNF-α receptors. The carrier vehicle in the composition of the invention is preferably a viscous formulation, and most preferably, sodium hyaluronate for application to the corneal surface, to promote a longer residence time for the therapeutic agent at the affected site of the patient.

Preferably, the method of the invention is used to prolong transplant survival in corneal allograft recipients or to support the establishment of stem cell transplants, such as retinal stem cell or limbal stem cell or amniotic membrane grafts, e.g., for treatment of ocular surface disease. The method of the invention would also be useful for therapeutic intervention in immunogenic inflammatory diseases of the cornea and ocular surface, such as keratoconjunctivitis sicca and other dry eye states including Sjögren's syndrome, allergic conjunctivitis and other atopic conditions of the ocular surface, corneal neovascularization, and immune or infectious keratitis states. Other disorders treatable by the methods of the invention include retinal

disorder or degeneration, age-related macular degeneration and degeneration of ganglion cells, as in glaucoma. In addition, the method of the invention would be useful for suppressing diseases such as uveitis and post-surgical inflammation in intraocular compartments (e.g., anterior chamber or vitreous cavity). Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows that local treatment with soluble TNF receptor I (sTNFRI) inhibits Langerhans cell (LC) migration into the central cornea of C57Bl/6 mice at 24h, 72h and 1wk after intracorneal injection of 1 μg/ml TNF;

Fig. 2 shows that treatment with subconjunctival injections of soluble TNF receptor I (sTNFR-I) inhibits Langerhans cell (LC) migration into the central cornea of C57Bl/6 mice 1 week after corneal cauterization or intracorneal injection of 1 μg/ml IL-1;

Fig. 3 shows that topical treatment with soluble TNF receptor I (sTNFR-I) drops inhibits Langerhans cell (LC) migration into the central cornea of C57Bl/6 mice after corneal cauterization;

Fig. 4 shows that the number of limbal Langerhans cells (LC) in C57Bl/6 mice corneas 24h, 72h and 1 wk after intracorneal injection of 1 μg/ml TNF-α is not changed by local treatment with soluble TNF receptor I (sTNFR-I);

Fig. 5 shows the fate of minor H-disparate corneal transplants following treatment with topical sTNFR-I or vehicle;

Figs. 6A and 6B show corneal neovascularization scores in minor-H-disparate allografts treated with topical control vehicle (A) or sTNFR-I (B);

Fig. 7 is an autoradiogram showing chemokine gene expression after corneal transplantation and treatment with sTNFRI; and

Figs. 8A and 8B show densitometry of RANTES (A) and MIP1β (B) gene expression after corneal transplantation.

DESCRIPTION OF PREFERRED EMBODIMENTS OF THE INVENTION

sTNFRI is a very promising agent for use in corneal transplantation or to support the establishment of stem cell transplants such as retinal stem cell and limbal stem cell grafts, both because of its efficacy as demonstrated in the experiments described below and its putative value over existing therapy, which has well-known side-effects and complications. In addition, the very significant dampening of the inflammatory response observed suggests that treatment with sTNFRI and other antagonists of TNF-α can be applied to a wide variety of ocular immune and inflammatory disorders.

TNF-α antagonism, e.g., via use of sTNFRI, can suppress immunogenic inflammation, as demonstrated using the corneal transplant model described below. Other appropriate therapeutic agents in the methods of the invention include, as well as sTNFRI, sTRFRII or a portion of either receptor, or a fusion protein comprising sTNFRI or sTRFRII or portions thereof. An exemplary fusion protein is the etanercept ENBREL™ (Immunex), which consists of an antibody constant region linked to the soluble portion of TNFRII. In the eye, local administration of a therapeutic agent described herein can include non-transplant therapeutic uses such as treatment of allergic and hypersensitivity disorders of the ocular surface, burns, infections, dry eye disorders, and chronic inflammatory states that may lead to scarring or fibrosis of the cornea and ocular surface. In addition, the observed decrease in neovascularization post-therapeutic treatment described herein suggests that

sTNFRI therapy can be useful to support conditions leading to angiogenesis. The method of the invention is also appropriate for treatment (or prophylaxis of recurrence) of intraocular inflammatory disorders such as autoimmune or infectious uveitis, post-traumatic or post-surgical inflammation, or idiopathic uveitides.

For any indication, the therapeutic composition should be administered according to the method of the invention by the most appropriate route, e.g., by topical application or by intraocular injection (e.g., into the anterior chamber or irrigation at the time of surgery). In addition to sodium hyaluronate, other vehicles may be used to increase drug delivery to the surface epithelium. Sustained release formulations, e.g., with use of biodegradable or non-degradable biocompatible polymers, or simple irrigation of these agent(s) at the time of surgery, can be used for intraocular delivery of sTNFRI to subjects.

Other candidate TNF-α antagonists that might be useful in the methods of the invention, as described above, can be tested for effectiveness using one of the assays described herein (e.g., measuring the extent of corneal inflammation, graft survival or Langerhans cell migration) and the results compared to those obtained with sTNFRI.

The dosage of sTNFRI used in the experiments described herein was relatively high in order to determine the maximum positive effect of treatment. However, sTNFRI should to be able to exert its suppressive effect over a wide dose range. Optimal dosage and appropriate modes of administration for each of the conditions delineated above can be determined by conventional protocols. For example, in the case of corneal transplantation, other doses ranging between 20ng/ml - 2mg/ml will additionally be tested and the endpoints described above (e.g., effect on corneal

inflammation, graft longevity or Langerhans cell migration) for the tested dosage will be compared to those obtained using the current doses described herein. It is to be expected that an appropriate concentration of a TNF-α antagonist in a vehicle for local administration to a human patient will be in the range of 20ng/ml to 50mg/ml.

The following examples are presented to illustrate the advantages of the present invention and to assist one of ordinary skill in making and using the same. These examples are not intended in any way otherwise to limit the scope of the disclosure.

EXAMPLE I

TNF-α induction of LC migration is suppressed by sTNFRI

It has been shown that Langerhans cell migration into the central cornea is significantly reduced in animals lacking one or both TNF receptors. Moreover, TNF-α injection into the central corneal stroma is able to induce centripetal LC migration (Dekaris, 1999). Murine eyes injected with TNF-α were treated with sTNFRI drops four times per day. Both early (24 and 72 hours) and late (1 week) LC migration into the central cornea was significantly reduced as compared to vehicle treated eyes (p=0.005, 0.024, 0.002, respectively). Even a stronger effect was recorded after administration of subconjunctival sTNFRI injections once per day (p=0.0013, 0.0005, 0.0005 at 24h, 72h and 1 wk, as compared to controls), as shown in Fig. 1. Although subconjunctival application of sTNFRI seemed to have higher a potential to reduce LC migration as compared to sTNFRI drops, no statistically significant difference between these two methods was recorded (p=0.9, 0.4, 0.1, for 24h, 72h and 1wk, respectively).

These results showed that sTNFRI can successfully suppress LC migration caused by intracorneal TNF-α injection. Next, the question as to whether LC chemotaxis caused by other stimuli (such as thermal cautery and intracorneal IL-1α injection) can be also modified by sTNFRI treatment was investigated. Using a previous model, it has been shown that both topical (drops) application and subconjunctival injection treatment modalities are equally effective, with a slightly better response to subconjunctival treatment. Therefore, in testing the efficacy of sTNFRI treatment in other experimental models, subconjunctival sTNFRI injections were used as the treating modality. Mice corneas were either a) cauterized or b) given an intrastromal IL-1α injection. Animals from each group were randomly divided in two subgroups, one receiving subconjunctival sTNFRI injections and one receiving vehicle only. Corneas were harvested after 1 week (the time necesary for LC migration to occur in this model (Dana et al., 1998)), and the number of LC in the central cornea was calculated. Eyes treated with subconjunctival sTNFRI injections had a significantly lower number of central LC, as compared to vehicle-treated eyes ($p=0.0027$ for cauterization and $p=0.001$ for IL-1α injection, see Fig. 2).

To test the efficiency of sTNFRI treatment for a prolonged time, mice corneas were cauterized and treated with sTNFRI drops. Subconjunctival injections were not used because of technical difficulties of performing 14 consecutive injections under mice conjunctiva, while drops were very easy to apply for a period of 2 weeks. sTNFRI again was shown to be successful in inhibiting LC migration, as compared to vehicle treated eyes ($p=0.0027$ for 1, and $p=0.0014$ for 2 weeks, Fig. 3).

Langerhans cells moving into the central cornea are recruited from the corneal periphery (limbus) where they

are normally situated. Therefore, the effect of sTNFRI treatment on the number of limbal LC in animals receiving intracorneal TNF-α injection was also examined. As shown in Fig. 4, there was no significant difference in limbal LC number between sTNFRI- (drops or subconjunctival injections alike) and vehicle-treated eyes. Bearing in mind that in non-treated animals significant amounts of LC are migrating into the central cornea after TNF-α injection, their number at the limbus should be decreased unless additional LC are recruited from the perilimbal vasculature following stimulation of the cornea. In sTNFRI treated eyes, the number of LC migrating into the cornea was observed to be decreased in comparison with non-treated eyes (controls). Therefore, if no additional LC were recruited in control eyes, LC the number at the limbus of treated eyes should be even higher then in controls. However, the number of LC in sTNFRI treated eyes remained equal to those in the controls. These results suggest not only that additional LC are recruited from the perilimbal vasculature in control eyes, but also that in sTNFRI-treated eyes LC, migration into the limbus is also suppressed. sTNFRI could, therefore, play a role even in the recruitment of LC into the limbal area (Fig. 4).

These data suggest that sTNFRI is an effective TNF-antagonist in a variety of *in vivo* models. The administration of sTNFRI drops was also effective to suppressing LC migration for a prolonged time (up to 2 weeks), showing that the suppressive effect of the treatment can be maintained throughout the studied time-period.

Possible strategies to modulate immune responses in the cornea and anterior eye segment are becoming increasingly interesting because of the well-known toxic side-effects of currently available immunosuppressive agents. Data from this study suggest that topical

treatment with sTNFRI can successfully suppress the migratory capacity of corneal Langerhans cells. The critical role of Langerhans cells in the abrogation of corneal immune privilege, initiation of immune responses and promotion of corneal graft rejection makes them an attractive target for future anti-inflammatory agents. Naturally occurring molecules such as sTNFRI will be invaluable as new immunosuppresive agents.

MATERIAL AND METHODS

Mice and anaesthesia. Six to eight-week old C57Bl/6 male mice were bred in the Schepens Eye Research Institute Animal Colony. All animals were treated according to the Statement for the Use of Animals in Ophthalmic and Vision Research by the Association for Research in Vision and Ophthalmology. Each animal was anesthetized with an intramuscular injection of 3-4 mg of ketamine and 0.1 mg of xylazine before surgical procedures. Experiments were performed on 10 murine corneas and replicated once.

Thermal cautery of the corneal surface. Mice were anesthetized and placed under the operating microscope. Using the tip of a hand-held cautery, five burns were applied to the central 50% of the cornea to induce centripetal LC migration (Williamson, et al., 1987). Mice were randomized to receive: sTNFRI drops, subconjunctival injections of sTNFRI or sodium hyalorunate (vehicle for sTNFRI, controls). All treatments were started immediately following surgery and applied throughout the study period. One and two weeks after cauterization, which correlates with the significant LC migration response in this model (Dana, et al., 1998), corneas were harvested and LC enumeration was performed as detailed below.

Intracorneal cytokine injections. A microsurgical blade (Superblade 30°, Kabi Pharmacia Ophthalmic Inc, USA) was used to make a horizontal 50% thickness intrastromal incision in the central cornea. After forming a tunnel in the stromal tissue, cytokine was injected intrastromaly by use of a 33-gauge needle (Delasco, Tokyo, Japan). Recombinant murine TNF-α (1 µg/ml, R&D Systems, Minneapolis, MN) or recombinant murine IL-1α (1 µg/ml, R&D Systems, Minneapolis, MN) were injected. At specified time points, corneas were harvested and LC enumeration was performed as described below.

Langerhans cells enumeration. Langerhans cells were enumerated in whole corneal epithelial sheets by use of indirect immunofluorescence assay, as described previously (Dana, et al., 1998). Briefly, at 24 h, 72 h, and 1 and 2 weeks following corneal stimulation (cautery or intracorneal cytokine injection) murine eyes were collected and the corneas were dissected. Corneas were placed in 20mM EDTA buffer and incubated for 30-40 minutes at 37°C. The epithelium was detached and washed in PBS at room temperature. Epithelial sheets were fixed with 95% alcohol for 30 minutes. After washing in PBS two times for 10 minutes, epithelial sheets were incubated with 1/15 diluted primary anti-murine Ia^b Ab for 45 minutes at 37°C. Negative controls bypassed this step. Epithelial sheets were washed twice in PBS for 10 minutes and incubated with 1/10 diluted fluorescein isothiocyanate-labeled goat anti-mouse secondary Ab for 30 minutes at 37°C (Pharmingen, San Diego, CA). Samples were mounted on slides and immediately examined under the fluorescent microscope. Langerhans cells were then enumerated using a square ocular grid.

Application of sTNFRI. Drops: One drop of PEG-sTNFRI (15-30 mg/ml of polyethylene glycolated sTNFRI) diluted in 0.2% sodium hyaluronate was applied four

times a day (Amgen, Inc., Thousand Oaks, CA.), starting on the day of corneal stimulation and continued until harvesting of the corneal tissue. Control eyes were treated with 0.2% sodium hyaluronate only. Subconjunctival injections: PEG-sTNFRI powder was diluted in phosphate-buffered saline to a concentration of 15-30 mg/ml and administered once per day through a 30-gauge needle. Solutions for injections were freshly prepared on a daily basis.

Statistical analysis. Comparison of the mean number of Langerhans cells between different mouse strains, as well as between treatment protocols, was made using the Student's *t* test.

EXAMPLE II

Prophylactic Administration of Topical sTNFRI Enhances Orthotopic Corneal Allograft Survival

In this study, the effect of topical soluble TNFR-I on the survival of minor H-disparate corneal transplants was investigated. A total of 50 corneas from B10.D2 mice were transplanted orthotopically to 50 Balb/c mice, of which 30 were randomly selected to receive topical sTNFR1 treatment and 20 to obtain 0.2% HA vehicle treatment. As shown in Fig. 5, corneal transplants treated with vehicle displayed a survival rate of only 55% at week 4 and 40% at week 8. In contrast, corneal grafts treated with sTNFR1 exhibited improved survival rates of 86.2% and 78.4% at week 4 and 8, respectively. The enhancement in corneal allograft acceptance by topical sTNFR1 treatment is statistically significant ($P = 0.0045$).

Because postkeratoplasty neovascularization facilitates the expression of immunity, it was important to examine whether treatment with sTNFR1 imposes any effect on corneal neovascularization (NV) scores. As shown in Fig. 2, corneas treated with sTNFR1 had a

similar corneal NV score distribution pattern to that in corneas treated with vehicle ($P > 0.05$), as observed over 8 weeks following transplantation. The exception is week 3, in which sTNFR1 showed a marginal angiostatic effect; 60% of the treated corneas had a NV score > 2 whereas only 27% of the corneas in the vehicle-treated group had a NV score >2 ($P = 0.047$).

Corneal allograft rejection is pathologically characterized by leukocytic infiltration into the graft stroma and adherence of mononuclear cells to the donor corneal endothelium. Consistently, as is described below, increased expression of mRNA for selective chemokines of the CXC (α) and CC (β) families is associated with corneal allograft rejection. The CXC (α) chemokine family, which includes interferon-γ inducible protein-10 (IP-10), interleukine-8 (IL-8), and macrophage inflammatory protein-2 (MIP-2), mediate recruitment of neutrophils, while CC chemokines, such as regulated upon activation normal T cell expressed and secreted (RANTES), eotaxin, MIP-1α, MIP-1β, monocyte chemoattractant protein-1 (MCP-1), and T-cell activation gene 3 (TCA3), are primarily involved in recruitment of immune cells such as antigen-presenting and T cells (Luster et al., 1988; Ward et al., 1998). Because TNF-α is one of the main stimuli for secretion of a wide array of chemokines, the influence of sTNFR-I on gene expression of the above chemokines was also examined after corneal transplantation.

Fig. 3 shows the mRNA levels of chemokines in a ribonuclease protection assay autoradiograph, and Fig. 4A and 4B shows densitometric quantification of RANTES and MIP1β. Normal control eyes expressed a significant level of Eotaxin and marginal levels of RANTES as well as lymphotactin. Eyes bearing accepted or rejected corneal grafts in vehicle-treated mice expressed similar mRNA levels of lymphotactin, RNATES, Eotaxin, MIP1β,

MIP2 and MCP1. Compared with naive control animals, both vehicle-treated groups significantly overexpressed RANTES and MIP1β. sTNFR1-treated eyes expressed RANTES, Eotaxin, MIP1β, and MIP2. Compared with the two vehicle-treated groups, there is a significant decrease in RANTES mRNA expression (Fig. 4A, P = 0.0002, P = 0.0014). In addition, the MIP1β mRNA level was suppressed by sTNFR1 treatment (Fig. 4B, P = 0.0435). The mRNA levels of RANTES and MIP1β in sTNFR1-treated eye were indistinguishable from that in naive control animals. Comparison of all other chemokine mRNA levels among vehicle-treated and sTNFR1-treated eyes did not show any statistical difference.

Current preventive and therapeutic regimens for corneal transplant rejection in human are associated with significant complications. Hence, it is desirable to devise intervention strategies that can prolong graft survival by specifically targeting molecules that mediate the immunogenicity of the allotransplant. These data indicate that local neutralization of TNF-α activity is an effective modality for suppressing TNF-α-mediated processes in the context of corneal transplantation.

MATERIALS AND METHODS

Mice. Adult BALB/c mice were purchased from Taconic Farms, Inc. (Germantown, NY), and adult B10.D2 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and used as experimental subjects or corneal graft donors between 8 and 10 weeks of age. All animals were treated according to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Pharmacological strategy. One drop (5 μl) of each topical preparation was applied to BALB/c recipient eyes three times a day for the 8 weeks of the study, starting

from 24 hours after transplantation. The experimental medication was composed of 7.5-15 mg/ml polyethylene glycolylated truncated monomeric recombinant methionyl human soluble tumor necrosis factor receptor type I (sTNFR-I) in 0.2% sodium hyaluronate (Amgen, Inc., Thousand Oaks, CA) in PBS. Placebo-treated animals received the vehicle 0.2% sodium hyaluronate only.

Corneal transplantation. As described previously, (Dana et al., 1997) each recipient was deeply anesthetized with an intraperitoneal injection of 3 mg ketamine and 0.0075 mg xylazine before all surgical procedures. The central 2 mm of the donor cornea was excised and secured in recipient graft beds with eight interrupted 11-0 nylon sutures (Sharpoint; Vanguard, Houston, TX). Antibiotic ointment was applied to the corneal surface, and the lids were closed for 12 hours with an 8-0 nylon tarsorrhaphy. All grafted eyes were examined after 72 hours; no grafts were excluded from analysis because of technical difficulties. Transplant sutures were removed in all cases on day 7.

Evaluation of orthotopic corneal transplants. Grafts were evaluated for the signs of rejection by slitlamp biomicroscopy twice weekly over eight weeks. At each time point, the grafts were scored for opacity and neovascularization (NV). A previously defined and standardized scoring system was used to grade the degree of opacification from 0 to 5+ (0 = clear graft, 1 = minimal superficial opacity, 2+ = mild stromal opacity with pupil margin and iris vessels visible, 3+ = moderate stromal opacity with only pupil margin visible, 4+ = intense stromal opacity with the anterior chamber visible, 5+ = maximal corneal opacity with total obscuration of the anterior chamber). Grafts with an opacity score of 2+ or higher after 3 weeks were considered to be rejected; grafts with an opacity score of 3+ or higher at 2 weeks that never cleared were also

regarded as rejected. NV was graded between 0 and 8 based on the degree of centripetal ingrowth and quadrantic involvement of the neovessels (Sano et al., 1996).

Ribonuclease protection assay for chemokine expression. Total RNA was extracted by the single-step method using RNA-Zol-B (Tel-Test, Inc., Friendswood, TX). Eyes were enucleated five weeks after transplantation, homogenized, and centrifuged to remove cellular debris. The RNA pellets obtained from three eyes were resuspended in nuclease-free water and processed together as a group. Detection and quantification of murine chemokine mRNAs were accomplished with a multiprobe RPA system (PharMingen, San Diego, CA) as recommended by the supplier. Briefly, a mixture of [α-^{32}P] uridine triphosphate-labeled antisense riboprobes was generated from the chemokine template set mCK-5 (PharMingen). Probes for the following chemokine mRNA were used: Ltn, lymphotactin; RANTES, regulated upon activation normal T cell and secreted; Eotaxin, MIP, macrophage inflammatory protein; MCP, monocyte chemoattactant protein; IP-10, interferon-γ inducible protein-10kd; TCA3, T cell activation gene 3. Fifteen micrograms of total RNA was used in each sample. Total RNA was hybridized overnight at 56°C with 300 pg of the ^{32}P antisense riboprobe mixture. Nuclease-protected RNA fragments were purified by ethanol precipitation. After purification, the samples were resolved on 5% polyacrylamide sequencing gels. The gels were dried and subjected to autoradiography. Protected bands were observed after exposure of gels to x-ray film. Specific bands were identified on the basis of their individual migration patterns in comparison with the undigested probes. The bands were quantitated by densitometric analysis (Image: National Institutes of Health, Bethesda, MD) and were normalized to

glyceraldehide-3-phosphate dehydrogenase (GAPDH). All samples were analyzed in triplet.

Statistical analysis. The rates of corneal graft survival were plotted as Kaplan-Meier survival curves and compared by using a Logrank (Mantel-Cox) test. Comparison of chemokine expressions among vehicle- and sTNFR1-treated groups was analyzed by one way analysis of variance (ANOVA) text using Instat Biostatistics software (GraphPad Software, Inc.). Statistical significance was defined as a P value less than 0.05.

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While the present invention has been described in conjunction with a preferred embodiment, one of ordinary skill, after reading the foregoing specification, will be able to effect various changes, substitutions of equivalents, and other alterations to the compositions and methods set forth herein. It is therefore intended that the protection granted by Letters Patent hereon be limited only by the definitions contained in the appended claims and equivalents thereof.

CLAIMS

What is claimed is:

1. A method for prophylaxis or treatment of corneal transplant rejection comprising

providing a corneal transplant recipient patient; and

locally applying a therapeutic composition to an affected area of said patient, wherein said therapeutic composition comprises a therapeutically effective amount of a tumor necrosis factor-α antagonist in association with a pharmaceutically acceptable carrier vehicle for topical application.

2. A method for prophylaxis or treatment of an immunogenic inflammatory disease comprising

providing a patient suffering from or believed to be at risk from an immunogenic inflammatory disease of the eye; and

locally applying a therapeutic composition to an affected area of said patient, wherein said therapeutic composition comprises a therapeutically effective amount of a tumor necrosis factor-α antagonist in association with a pharmaceutically acceptable carrier vehicle for topical application.

3. A method for prophylaxis or treatment of an immunogenic inflammatory disease comprising

providing a patient suffering from or believed to be at risk from an immunogenic inflammatory disease of the eye; and

locally applying a therapeutic composition to an affected area of said patient, wherein said therapeutic composition comprises a therapeutically effective amount of an inhibitor of tumor necrosis factor-α dependent corneal Langerhans cell migration in association with a

pharmaceutically acceptable carrier vehicle for topical application.

4. The method of claim 1, claim 2 or claim 3 wherein said applying step is by topical application.

5. The method of claim 1 or claim 2 wherein said tumor necrosis factor-α antagonist in said therapeutic composition is a tumor necrosis factor receptor.

6. The method of claim 1 or claim 2 wherein said tumor necrosis factor-α antagonist in said therapeutic composition is sTNFRI or sTRFRII, or a portion thereof, or a fusion protein comprising sTNFRI or sTRFRII or a portion thereof.

7. The method of claim 3 wherein said inhibitor of tumor necrosis factor-α dependent corneal Langerhans cell migration in said therapeutic composition is sTNFRI or sTRFRII, or a portion thereof, or a fusion protein comprising sTNFRI or sTRFRII or a portion thereof.

8. The method of claim 1, claim 2 or claim 3 wherein said carrier vehicle in said therapeutic composition comprises sodium hyaluronate.

9. A method for prophylaxis or treatment of corneal transplant rejection comprising

providing a corneal transplant recipient patient; and

topically applying a therapeutic composition to an affected area of said patient, wherein said therapeutic composition comprises a therapeutically effective amount of sTNFRI in association with a pharmaceutically acceptable carrier vehicle for topical application, said vehicle comprising sodium hyaluronate.

10. An article of manufacture comprising packaging material and a therapeutic composition contained within said packaging material, wherein the therapeutic composition is therapeutically effective for prophylaxis or treatment of corneal transplant rejection and wherein the packaging material comprises a label that indicates that the therapeutic composition can be used locally for prophylaxis or treatment of corneal transplant rejection, and

wherein said therapeutic composition comprises a therapeutically effective amount of a tumor necrosis factor-α antagonist in association with a pharmaceutically acceptable carrier vehicle for local application.

11. An article of manufacture comprising packaging material and a therapeutic composition contained within said packaging material, wherein the therapeutic composition is therapeutically effective for prophylaxis or treatment of an immunogenic inflammatory disease of the eye and wherein the packaging material comprises a label that indicates that the therapeutic composition can be used locally for prophylaxis or treatment of an immunogenic inflammatory disease of the eye, and

wherein said therapeutic composition comprises a therapeutically effective amount of a tumor necrosis factor-α antagonist in association with a pharmaceutically acceptable carrier vehicle for local application.

12. An article of manufacture comprising packaging material and a therapeutic composition contained within said packaging material, wherein the therapeutic composition is therapeutically effective for prophylaxis or treatment of an immunogenic inflammatory disease of

the eye and wherein the packaging material comprises a label that indicates that the therapeutic composition can be used locally for prophylaxis or treatment of an immunogenic inflammatory disease of the eye, and

wherein said therapeutic composition comprises a therapeutically effective amount of an inhibitor of tumor necrosis factor-α dependent corneal Langerhans cell migration in association with a pharmaceutically acceptable carrier vehicle for local application.

13. The article of manufacture of claim 10 or claim 11 wherein, in said therapeutic composition, said tumor necrosis factor-α antagonist is a tumor necrosis factor receptor.

14. The article of manufacture of claim 10 or claim 11 wherein said tumor necrosis factor-α antagonist in said therapeutic composition is sTNFRI or sTRFRII, or a portion thereof, or a fusion protein comprising sTNFRI or sTRFRII or a portion thereof.

15. The article of manufacture of claim 12 wherein said inhibitor of tumor necrosis factor-α dependent corneal Langerhans cell migration in said therapeutic composition is sTNFRI or sTRFRII, or a portion thereof, or a fusion protein comprising sTNFRI or sTRFRII or a portion thereof.

16. The article of manufacture of claim 10, claim 11 or claim 12 wherein, in said therapeutic composition, said carrier vehicle comprises sodium hyaluronate.

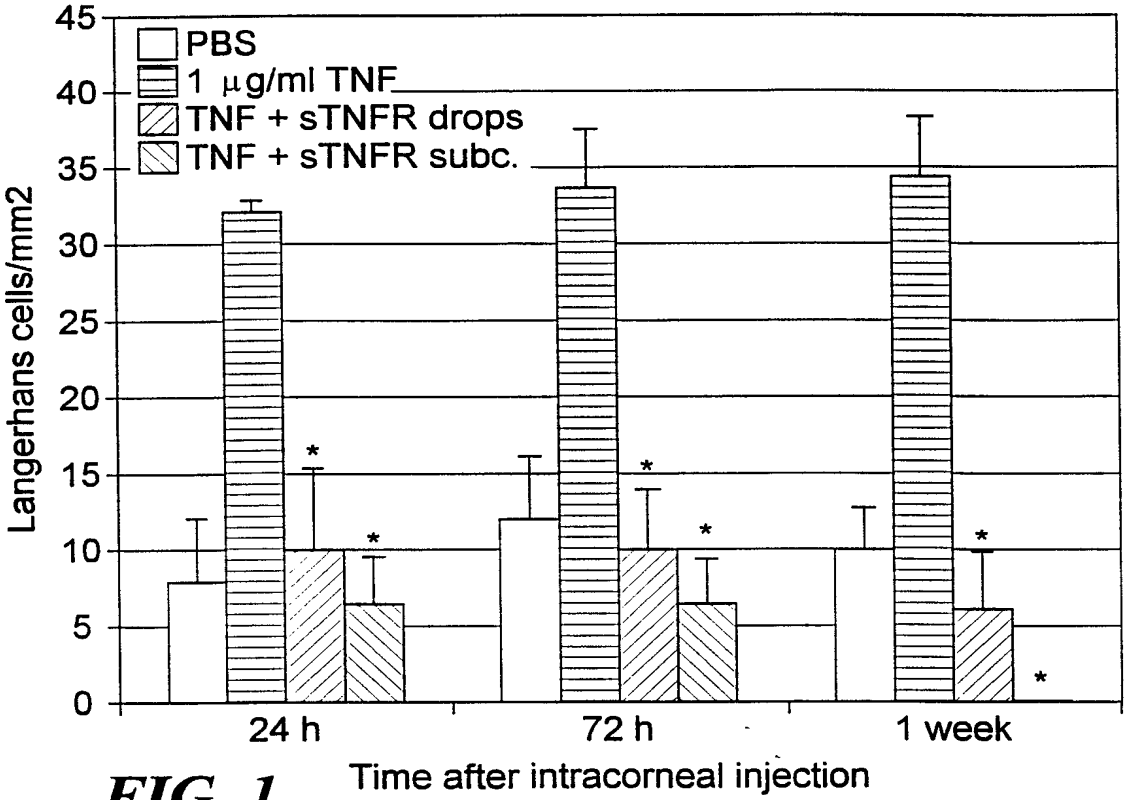
PBS
1 µg/ml TNF
TNF + sTNFR drops
TNF + sTNFR subc.
Langerhans cells/mm2
45
40
35
30
25
20
15
10
5
0
*
24 h
72 h
1 week
Time after intracorneal injection

FIG. 1

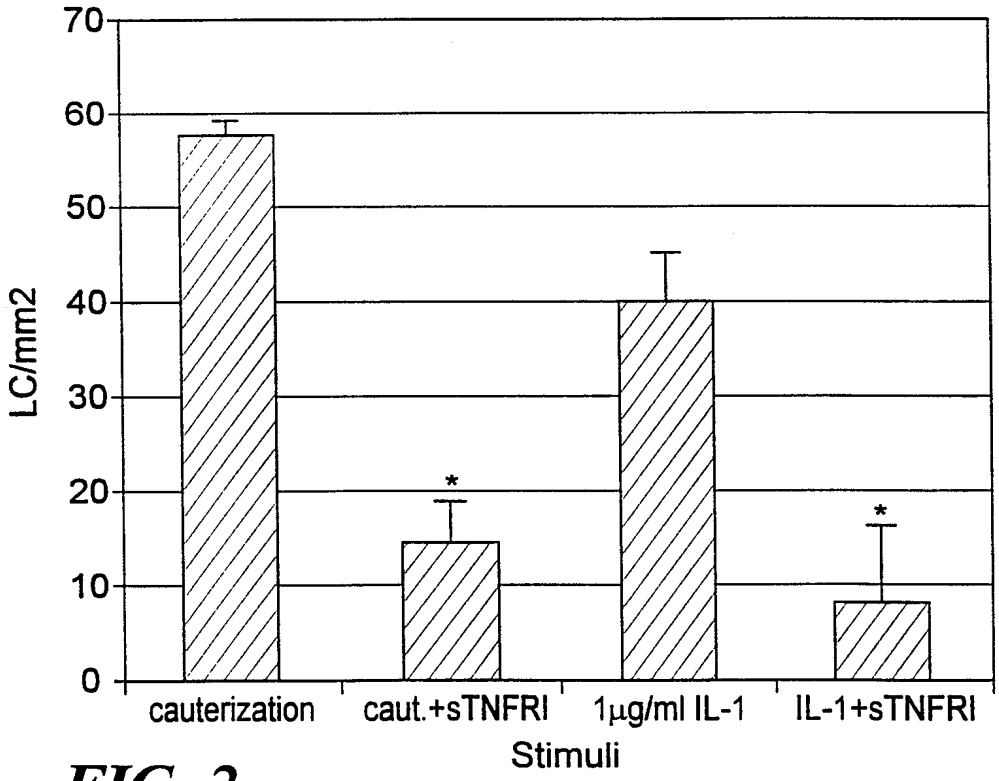
LC/mm2
70
60
50
40
30
20
10
0
*
cauterization
caut.+sTNFRI
1µg/ml IL-1
IL-1+sTNFRI
Stimuli

FIG. 2

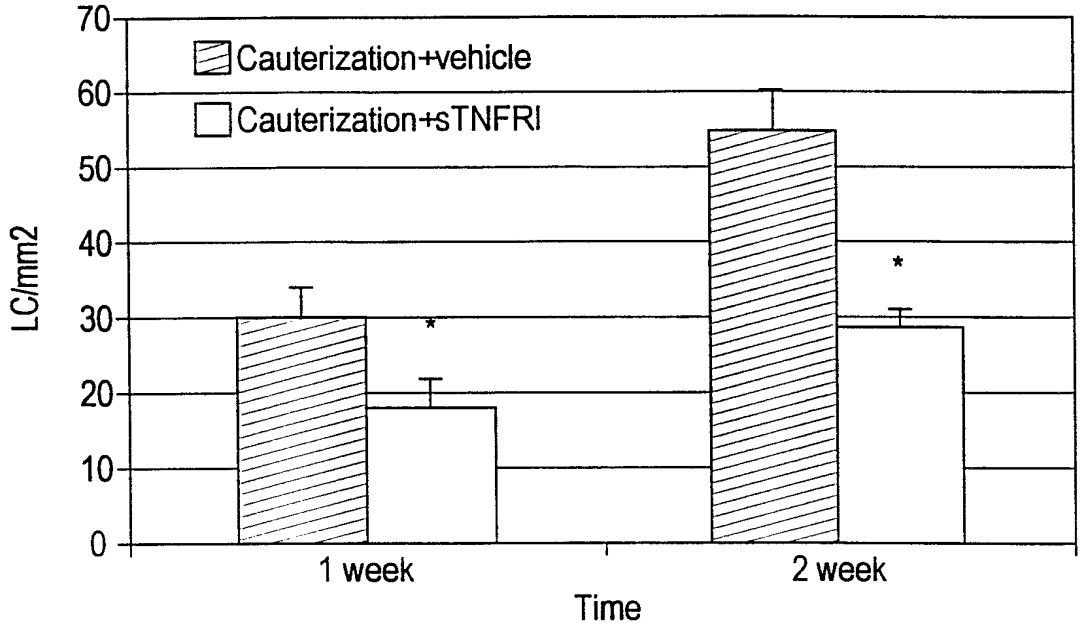
70
60
50
40
30
20
10
0
LC/mm2
Cauterization+vehicle
Cauterization+sTNFRI
*
*
1 week
2 week
Time

FIG. 3

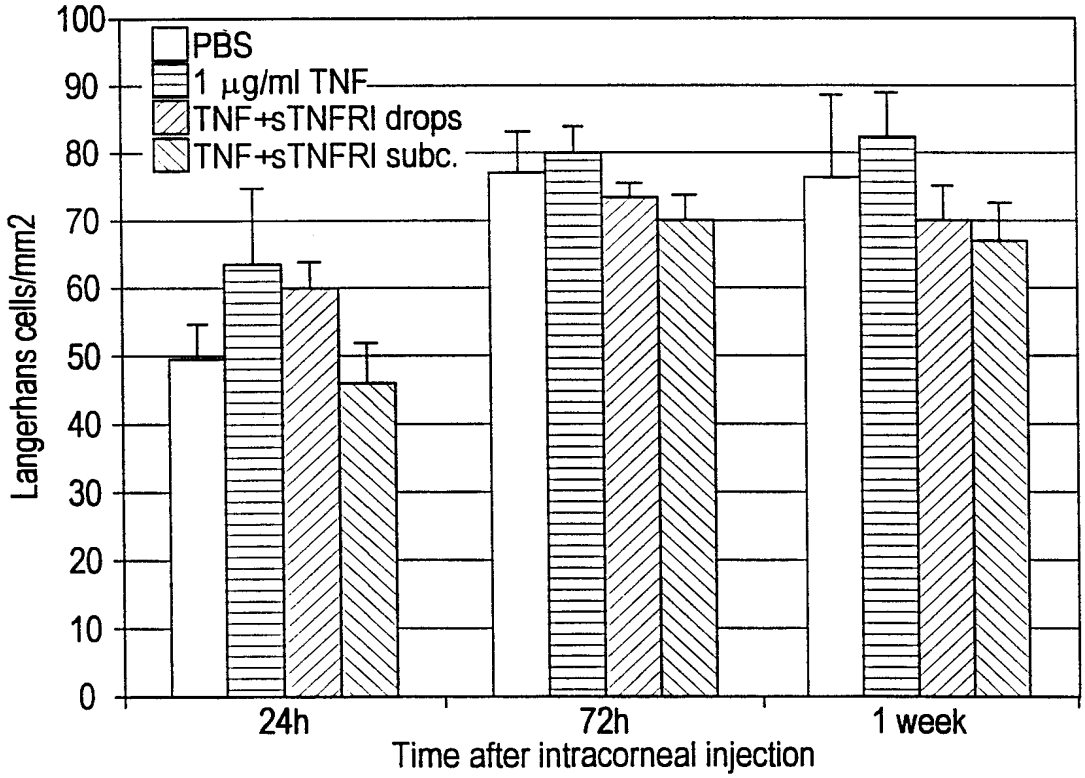
100
90
80
70
60
50
40
30
20
10
0
Langerhans cells/mm2
PBS
1 μg/ml TNF
TNF+sTNFRI drops
TNF+sTNFRI subc.
24h
72h
1 week
Time after intracorneal injection

FIG. 4

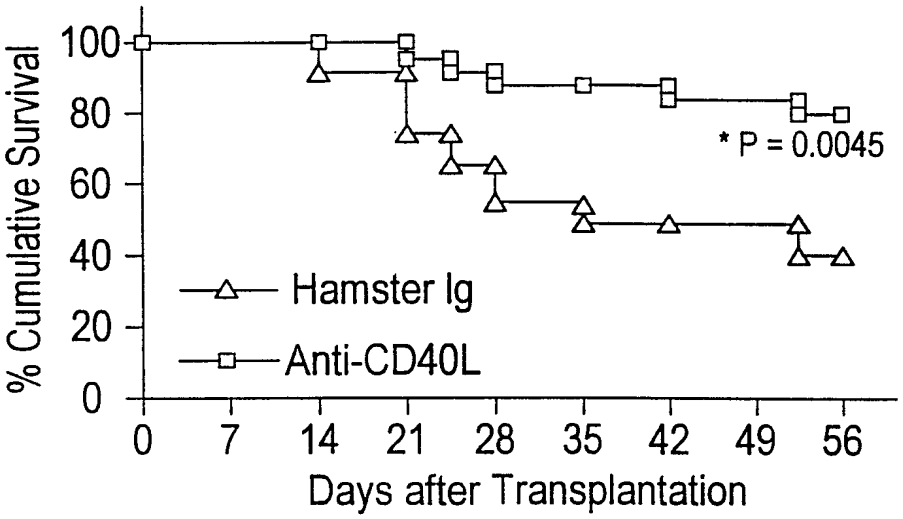

FIG. 5

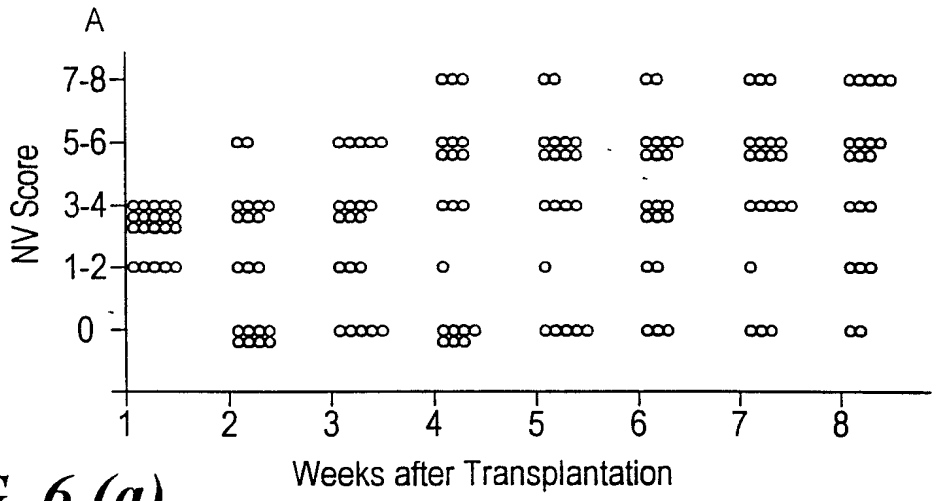

FIG. 6 (a)

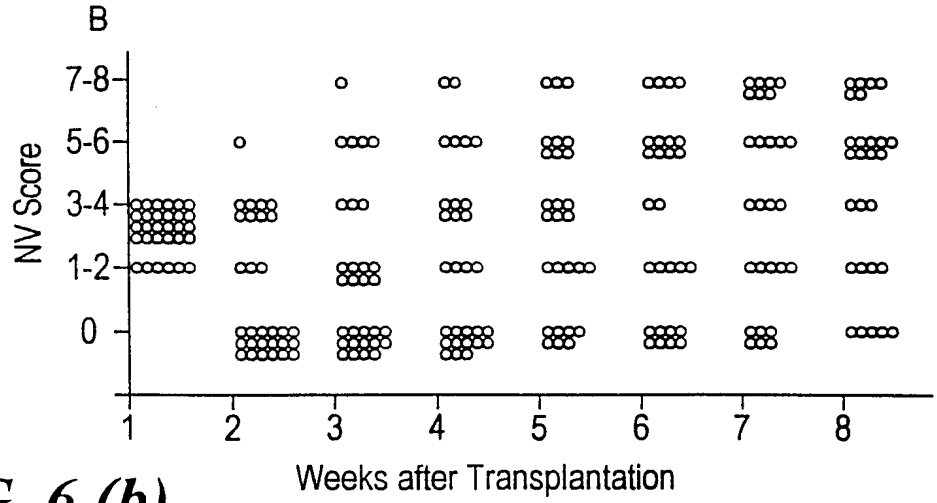

FIG. 6 (b)

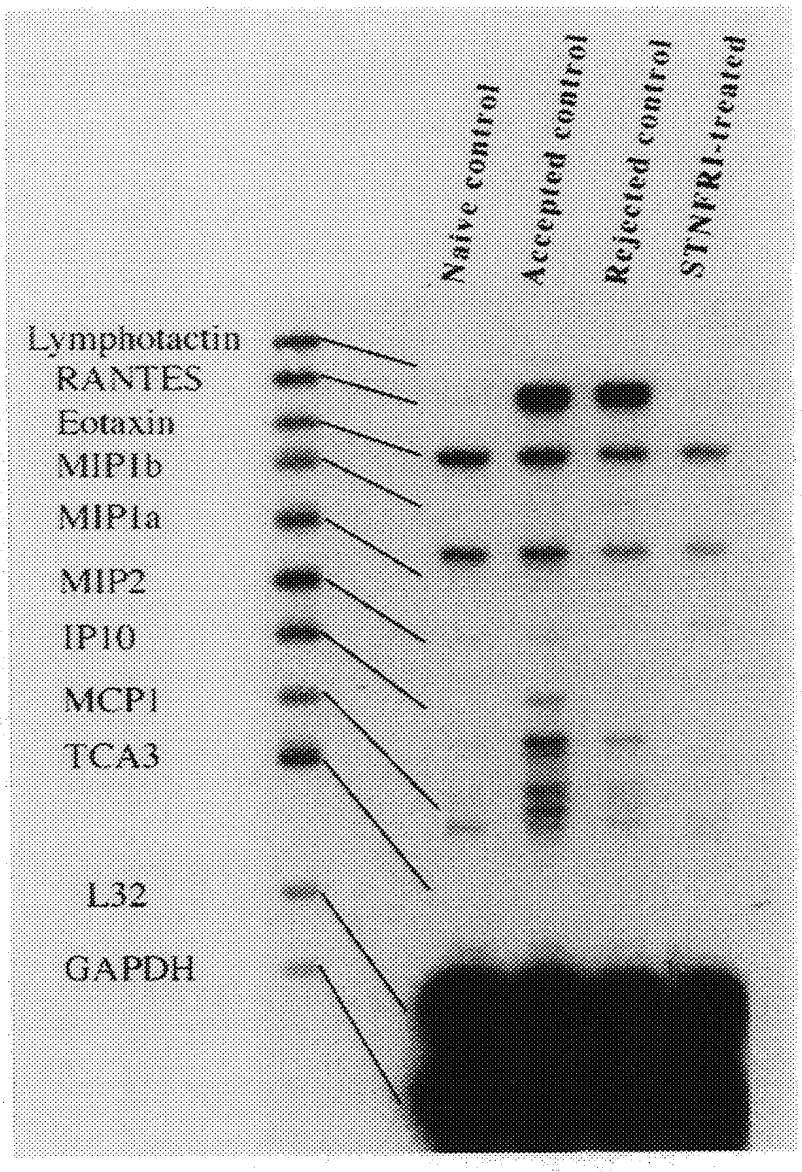
Naive control
Accepted control
Rejected control
STNFRI-treated
Lymphotactin
RANTES
Eotaxin
MIP1b
MIP1a
MIP2
IP10
MCP1
TCA3
L32
GAPDH

FIG. 7

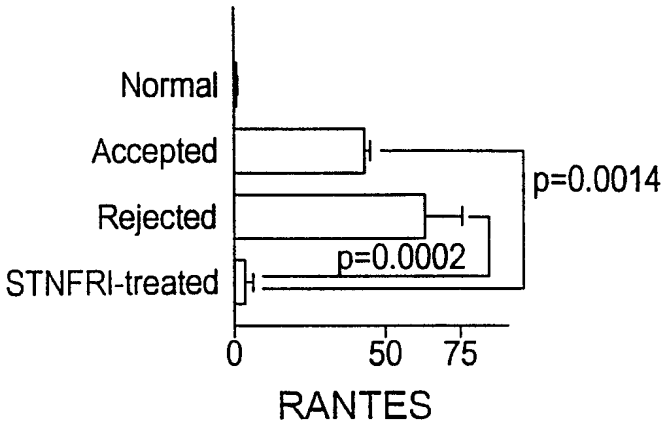
Normal
Accepted
Rejected
STNFRI-treated
p=0.0014
p=0.0002
0
50
75
RANTES

FIG. 8 (a)

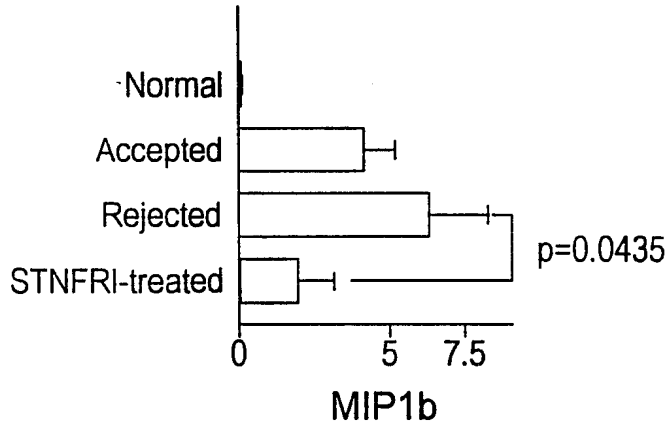
Normal
Accepted
Rejected
STNFRI-treated
p=0.0435
0
5
7.5
MIP1b

FIG. 8 (b)